

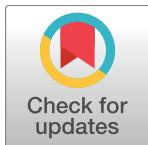
## RESEARCH ARTICLE

# Balancing anti-inflammatory and anti-oxidant responses in murine bone marrow derived macrophages

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## Abstract

### Rationale

The underlying pathophysiology of bronchopulmonary dysplasia includes a macrophage-mediated host response orchestrated by anti-inflammatory peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) and anti-oxidant nuclear factor (erythroid-derived 2)-like 2 (Nrf2). These have not yet been studied in combination. This study tested the hypothesis that combined inflammatory and oxidative stressors would interact and change PPAR $\gamma$ - and Nrf2-regulated gene expression and antioxidant capacity. Therefore, we investigated the effect of dual stimulation with lipopolysaccharide and hyperoxia in murine bone marrow-derived macrophages (BMDM).

### OPEN ACCESS

**Citation:** Nitkin CR, Bonfield TL (2017) Balancing anti-inflammatory and anti-oxidant responses in murine bone marrow derived macrophages. PLoS ONE 12(9): e0184469. <https://doi.org/10.1371/journal.pone.0184469>

**Editor:** Partha Mukhopadhyay, National Institutes of Health, UNITED STATES

**Received:** May 1, 2017

**Accepted:** August 24, 2017

**Published:** September 8, 2017

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**Data Availability Statement:** All relevant data are within the paper and its Supporting Information files.

**Funding:** Funding provided by the Rainbow Babies & Children's Foundation (<http://www.uhhospitals.org/rainbow/about/rainbow-foundation>) (CRN), the Little Giraffe Foundation (<http://www.littlegiraffefoundation.org/>) (CRN), and the David and Virginia Baldwin Fund (TLB). Dr. Nitkin's fellowship training is supported by NIH 5-T32-HD060537-07 (PI Richard J. Martin). The funders had no role in study design, data collection and

### Methods

Sub-confluent BMDM from wild-type C57BL/6J mice were treated with lipopolysaccharide (LPS) 1 $\mu$ g/mL for 2 hours followed by room air (21% oxygen) or hyperoxia (95% oxygen) for 24 hours. Taqman real time-polymerase chain reaction gene expression assays, total anti-oxidant capacity assays, and Luminex assays were performed.

### Results

Supernatants of cultured BMDM contained significant antioxidant capacity. In room air, LPS treatment decreased expression of PPAR $\gamma$  and Nrf2, and increased expression of tumor necrosis factor-alpha and heme oxygenase-1; similar findings were observed under hyperoxic conditions. LPS treatment decreased cellular total antioxidant capacity in room air but not in hyperoxia. Increased expression of sulfiredoxin-1 in response to hyperoxia was not observed in LPS-treated cells. Dual stimulation with LPS treatment and exposure to hyperoxia did not have synergistic effects on gene expression. Cellular total antioxidant capacity was not changed by hyperoxia exposure.

analysis, decision to publish, or preparation of the manuscript.

**Competing interests:** The authors have declared that no competing interests exist.

## Conclusions

Our hypothesis was supported and we demonstrate an interaction between inflammatory and oxidative stressors in a model system of bronchopulmonary dysplasia pathogenesis. The protective anti-oxidant effect of cell culture media may have protected the cells from the most deleterious effects of hyperoxia.

## Introduction

Bronchopulmonary dysplasia (BPD) is the major pulmonary morbidity of preterm birth, affecting up to 45% of very low birth-weight infants (less than 1500 grams) [1]. As many as 10% of these infants die and many experience neurodevelopmental and cognitive impairment [2]. A diagnosis of BPD more than doubles the cost of initial hospitalization [3] and places significant financial and emotional stresses on the family. Advances in neonatal care such as gentle ventilation, judicious use of steroids, caffeine, vitamin A, and appropriate nutrition have improved many outcomes in premature infants [4]. However, there is currently no cure for BPD, and two of the most significant pathogenic factors, infection and supplemental oxygen therapy [5], are often unavoidable.

The underlying pathophysiology of BPD is multifactorial but includes inflammation and oxidative stress which lead to airway remodeling, alveolar simplification, and pulmonary vasculature abnormalities [5,6]. BPD research has historically focused on inflammation, but the antioxidant response is also a significant contributor [7]. Our study sought to understand the roles of both inflammation and oxidative stress, including the two transcription factors peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) [8] and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) [9], which coordinate the anti-inflammatory and anti-oxidant responses, respectively, as well as the downstream effectors tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) (pro-inflammatory), sulfiredoxin (Srxn1) (anti-oxidant), and heme oxygenase-1 (Hmox1) (anti-inflammatory and anti-oxidant). To investigate the molecular pathways responsible for the cellular response to the pathogenic stimuli important in BPD, we have developed an *in vitro* model of BPD using murine bone marrow-derived macrophages (BMDM) treated with lipopolysaccharide (LPS), an inflammatory stimulus, and hyperoxia, an oxidative stress. This study tested the hypothesis that combined inflammatory and oxidative stressors would interact and change PPAR $\gamma$ - and Nrf2-regulated gene expression and antioxidant capacity. We present data which suggests an interaction between the molecular pathways responsive to inflammatory and oxidative stressors.

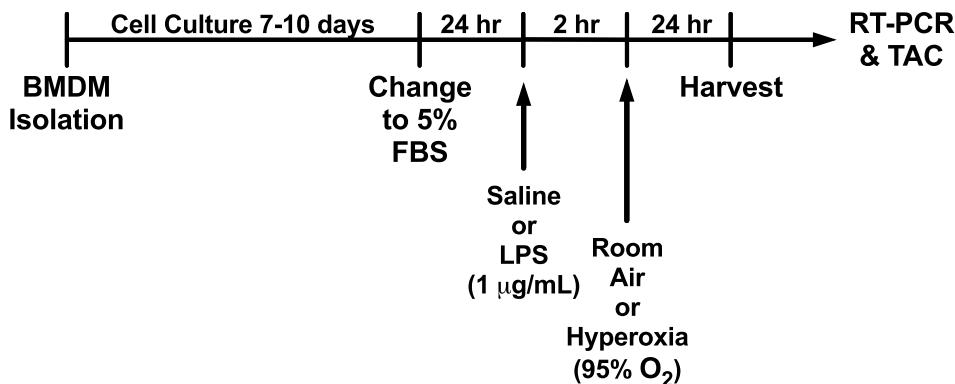
## Materials and methods

### Animals

The experimental design is outlined in Fig 1. All studies were submitted to and approved by the Case Western Reserve University Institutional Animal Care and Use Committee (protocol 2014–0093). No surgeries were performed on living animals, and BMDM were isolated from mice euthanized with carbon dioxide and cervical dislocation.

### Cell culture

**BMDM cell culture.** BMDM were generated from bone marrow from tibia and femur of six-week-old, wild-type C57BL/6J mice as previously described [10–12]. Briefly, bone marrow



**Fig 1. Experimental design.** BMDM were isolated from wild-type C57BL/6J mice and grown to subconfluence, then were treated with LPS or saline, followed 2 hours later by hyperoxia or room air, and were harvested 24 hours later.

<https://doi.org/10.1371/journal.pone.0184469.g001>

was collected and plated in six-well tissue culture plates (Corning, Corning, New York, United States). BMDM were cultured in RPMI 1640-based media (Fisher Scientific, Suwanee, Georgia, United States) containing 10% heat-inactivated fetal bovine serum (Life Technologies, Waltham, Massachusetts, United States), 1% Penicillin-Streptomycin-Glutamine (Life Technologies), and 20% spent L929-cell conditioned media as a source of M-CSF [10], exchanging media every 48–72 hours. This standard cell culture media was exchanged for an equal volume of media supplemented with 5% instead of 10% FBS twenty-four hours prior to LPS/hyperoxia exposure. Following LPS/hyperoxia treatment, cells were manually removed by scraping, the suspension was centrifuged at 400 x g for 9 minutes, and cell pellet and supernatant were stored separately at -80°C.

**Hyperoxia treatment.** Cell culture plates were placed in a 5.7 L modular incubator chamber (Billups-Rothenberg, Del Mar, California, United States) which was then flushed with a gas mixture of 95% oxygen and 5% carbon dioxide (Airgas Corporation, Radnor Township, Pennsylvania, United States) until oxygen saturation reached 95±2% as measured with a MaxO<sub>2</sub>+AE oxygen analyzer (Maxtec, Salt Lake City, Utah, United States). This prepared chamber was placed within a standard cell culture incubator at 37°C. Control cells were kept in a standard cell culture incubator with room air (21% oxygen) supplemented with 5% carbon dioxide. Of note, hyperoxia treatment, with or without LPS treatment, did not cause any cell death or observable cellular stress, as observed from the microscopic evaluation of cell confluence.

**LPS treatment.** Aliquots of LPS (Sigma-Aldrich, St. Louis, Missouri, United States) were stored at -80°C and diluted with phosphate buffered saline (Genesee Scientific, San Diego, California, United States), then were applied to cells for a final concentration of 1 µg/mL. Control cells were treated with equal volumes of phosphate buffered saline. Of note, LPS treatment, with or without hyperoxia treatment, did not cause any cell death or observable cellular stress, as observed from the microscopic evaluation of cell confluence.

## Gene expression

RNA was isolated from cells with Ribozol reagent (Amresco, Solon, Ohio, United States) and cDNA was synthesized using qScript cDNA Synthesis Kits (VWR, Radnor, Pennsylvania, United States) and stored at -80°C. RNA and cDNA concentrations were determined by spectrophotometry (SpectraMax i3x, Molecular Devices, Sunnyvale, California, United States). cDNA was diluted in sterile nuclease-free water to which TaqMan Universal PCR Master Mix

and PCR primers (Life Technologies) were added. Pre-validated TaqMan primers spanning exon junctions were used to prevent detection of genomic DNA: assay ID number PPAR $\gamma$  (Mm01184322\_m1), TNF $\alpha$  (Mm00443258\_m1), Nrf2 (Mm00477784\_m1), Srxn1 (Mm00769566\_m1), and Hmox1 (Mm00516005\_m1). Samples were plated in a 96-well reaction plate, run on an Applied BioSystems 7300 Real Time PCR System, and analyzed using ABI 7300 Prism software and Microsoft Excel.

### Luminex ELISA

Cytokine levels from cell culture supernatants were assessed by Luminex assay (R&D Systems, Minneapolis, Minnesota) per manufacturer's instructions. Supernatants were centrifuged and stored at -80°C until evaluation for TNF $\alpha$ . Undiluted samples were then run on a Luminex Magpix according to the manufacturer's protocol.

### Total antioxidant capacity

Concentration of small molecule and protein antioxidants was measured using a Total Antioxidant Capacity Assay Kit (Sigma-Aldrich) per the manufacturer's instructions. Absorbance was measured by spectrophotometry (SpectraMax i3x). Cell culture media constituents (FBS, L929-cell conditioned media, RPMI media) were assayed individually, cell culture media without cells (RPMI supplemented with 5% FBS, 10% L929-cell conditioned media) treated with LPS or saline and/or 95% hyperoxia or room air was assayed, and supernatants from harvested BMDM which had been treated with LPS or saline and/or 95% hyperoxia or room air were assayed.

### Statistical analyses

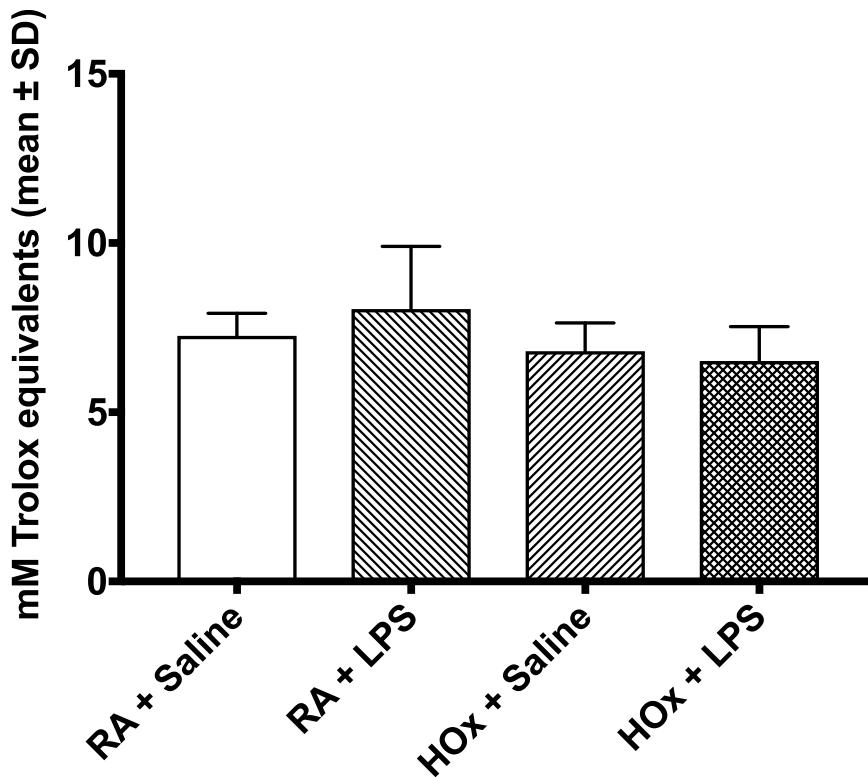
Gene expression data were normalized to GAPDH to obtain  $\Delta Ct$  values. Change in expression, relative to normoxia or saline controls as noted in the text, was calculated using the  $2^{-\Delta\Delta Ct}$  method [13]. Because Gaussian distribution could not be assumed, data were analyzed with Kruskal-Wallis test with Dunn's correction for multiple comparisons using GraphPad Prism 7.0c for Mac (GraphPad Software, La Jolla California USA). A P value less than 0.05 was considered statistically significant.

## Results

### Cell culture media has antioxidant activity

Because preliminary experiments did not reveal any significant effect of hyperoxia on gene expression, we hypothesized that the cell culture media might have antioxidant activity, protecting the cells from experiencing marked oxidative stress. Therefore, we measured total antioxidant capacity (TAC) of the cell-free culture media treated with LPS and hyperoxia, as well as FBS, L929-CM, and RPMI, plus supernatants from harvested BMDM.

We found that significant antioxidant activity was present in all components of the cell culture media (FBS 12.12 [SD 0.1669] mM Trolox equivalents, L929-conditioned media 6.966 [SD 0.074], and RPMI 1.789 [0.093]). Additionally, BMDM supernatant under all experimental conditions demonstrated robust TAC, greater than expected by simple addition of TAC of each of the constituents (Fig 2). However, we found no significant difference in TAC after treatment with LPS or hyperoxia. We thus sought to investigate transcriptional regulation of antioxidant genes within BMDM, but cautiously interpreted our findings, suspecting that the antioxidant activity present in the media would prevent the BMDM from "experiencing" oxidative stress.



**Fig 2. Total antioxidant capacity of BMDM supernatant.** Supernatant from BMDM treated with LPS or saline and hyperoxia (HOx) or room air (RA) was assayed for total antioxidant capacity. Values are presented as mM Trolox equivalents, an antioxidant standard. Values are mean  $\pm$  SD.

<https://doi.org/10.1371/journal.pone.0184469.g002>

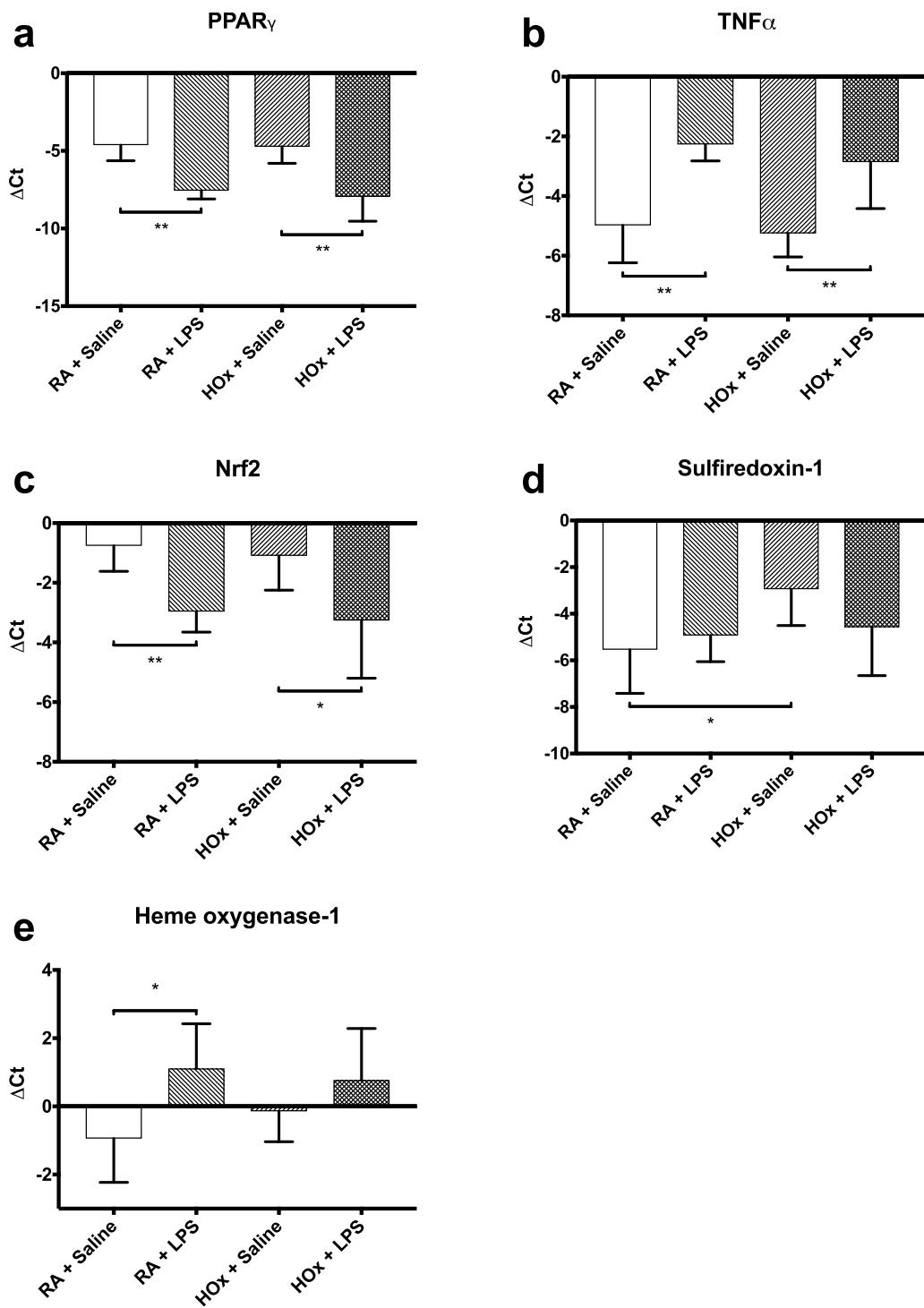
### LPS induces a pro-inflammatory phenotype

For our first gene expression studies, we examined the effect of LPS stimulation on inflammation-related gene expression in BMDM cultured under 21% oxygen (room air). Because FBS has antioxidant activity, we exchanged standard cell culture media containing 10% FBS with serum with reduced 5% FBS; we found a further reduction in FBS resulted in significant cell death which precluded further experiments.

In room air, LPS treatment induced a pro-inflammatory phenotype (Fig 3a and 3b), as evidenced by decreased PPAR $\gamma$  expression ( $\Delta Ct$  from -4.632 [SD 1.006] to -7.568 [SD 0.529],  $p = 0.002$ ) and increased TNF $\alpha$  ( $\Delta Ct$  from -4.983 [SD 1.253] to -2.27 [SD 0.553],  $p = 0.007$ ), relative to room air-exposed saline controls. This same decrease in PPAR $\gamma$  ( $\Delta Ct$  from -4.738 [SD 1.066] to -7.961 [SD 1.568],  $p = 0.002$ ) and increase in TNF $\alpha$  ( $\Delta Ct$  from -5.251 [SD 0.790] to -2.861 [SD 1.560],  $p = 0.008$ ), relative to saline controls, was observed in hyperoxia-exposed BMDM. As expected, LPS treatment also increased TNF $\alpha$  protein levels in room air and hyperoxia (Fig 4) (1.21 [SD 0.3] to 295.3 [SD 96.6] pg/mL in room air, and 1.11 [SD 0.4] to 257.9 [SD 114.4] in hyperoxia).

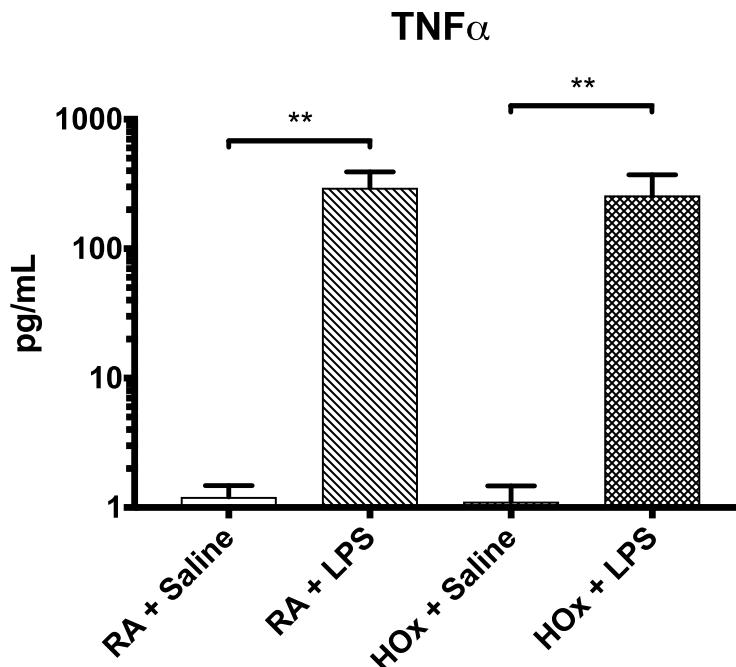
### Pro-inflammatory stimulation diminishes antioxidant gene expression

We then studied the effect of LPS stimulation on antioxidant-related gene expression (Fig 3c–3e). In room air, LPS treatment suppressed Nrf2 gene expression ( $\Delta Ct$  from -0.763 [SD 0.856] to -2.968 [SD 0.686],  $p = 0.002$ ) and increased Hmox1 expression ( $\Delta Ct$  from -0.945 [SD 1.284] to 1.113 [SD 1.310],  $p = 0.015$ ) relative to saline controls, but did not significantly impact



**Fig 3.  $\Delta Ct$  values for genes of interest in BMDM.** Cells treated with LPS or saline control, and placed in room air (RA) or hyperoxia (HOx).  $\Delta Ct$  values represent difference in threshold cycle between gene of interest and GAPDH. Values are mean  $\pm$  SD. \*  $p < 0.05$ , \*\*  $p < 0.01$ .

<https://doi.org/10.1371/journal.pone.0184469.g003>



**Fig 4. TNF $\alpha$  values in BMDM supernatant.** Cells were treated with LPS or saline control, and placed in room air (RA) or hyperoxia (HOx). TNF $\alpha$  levels of the cell culture supernatant were measured via Luminex ELISA. Values are mean  $\pm$  SD. \*\* p<0.01.

<https://doi.org/10.1371/journal.pone.0184469.g004>

Srxn1 gene expression ( $\Delta Ct$  from -5.545 [SD 1.870] to -4.934 [SD 1.122],  $p>0.999$ ). LPS stimulation also significantly decreased total antioxidant capacity by 11.2% ( $p = 0.005$ ) (Fig 5).

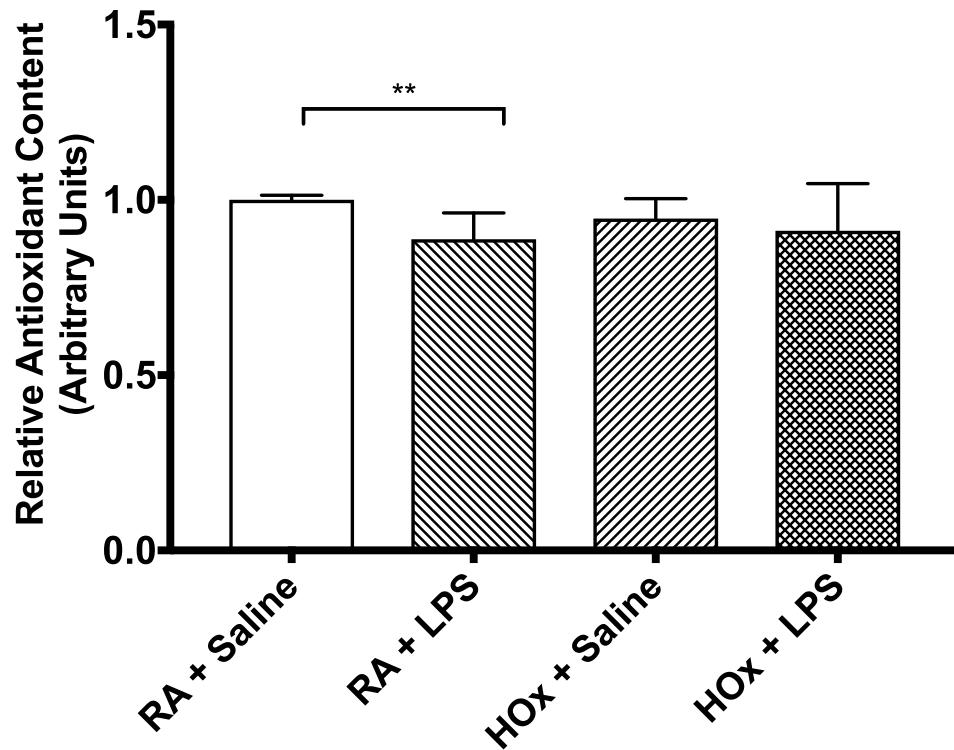
In hyperoxia, LPS treatment suppressed Nrf2 gene expression ( $\Delta Ct$  from -1.079 [SD 1.153] to -3.269 [SD 1.932],  $p = 0.046$ ) relative to saline controls, but had no effect on expression of Srxn1 ( $\Delta Ct$  from -2.947 [SD 1.560] to -4.582 [SD 2.074],  $p = 0.345$ ) or Hmox1 ( $\Delta Ct$  from -0.147 [SD 0.891] to 0.772 [SD 1.512],  $p = 0.391$ ). In hyperoxia, LPS stimulation did not significantly decrease total antioxidant capacity (94.7 vs 91.2%,  $p>0.999$ ) (Fig 5).

### Hyperoxia increases antioxidant gene expression

Our next experiment examined the effect of hyperoxia on gene expression in the absence of LPS (Fig 3c–3e). Hyperoxia treatment increased Srxn1 expression ( $\Delta Ct$  from -5.545 [SD 1.870] to -2.947 [SD 1.560],  $p = 0.022$ ) relative to room air controls, with no significant effect on expression of PPAR $\gamma$  ( $\Delta Ct$  from -4.632 [SD 1.006] to -4.738 [SD 1.066]), TNF $\alpha$  ( $\Delta Ct$  from -4.983 [SD 1.253] to -5.251 [SD 0.790]), Nrf2 ( $\Delta Ct$  from -0.763 [SD 0.856] to -1.097 [SD 1.153]), or Hmox1 ( $\Delta Ct$  from -0.9452 [SD 1.284] to -0.1467 [SD 0.891]) ( $p>0.999$  for all). Hyperoxia treatment did not significantly alter total antioxidant capacity (100 vs 94.7%,  $p = 0.313$ ) (Fig 5).

### LPS and hyperoxia treatment is not synergistic

Finally, we examined the effect of hyperoxia on gene expression in the presence of LPS (Fig 3c–3e). Hyperoxia treatment had no significant effect on expression of PPAR $\gamma$  ( $\Delta Ct$  from -7.568 [SD 0.529] to -7.961 [SD 1.568]), TNF $\alpha$  ( $\Delta Ct$  from -2.27 [SD 0.553] to -2.861 [SD 1.560]), Nrf2 ( $\Delta Ct$  from -2.968 [SD 0.686] to -3.269 [SD 1.932]), Srxn1 ( $\Delta Ct$  from -4.934 [SD 1.122] to -4.582 [SD 2.074]), or Hmox1 ( $\Delta Ct$  from 1.113 [SD 1.310] to 0.772 [SD 1.512])



**Fig 5. Total antioxidant capacity of BMDM homogenate.** Relative total antioxidant capacity per protein content, normalized to room air (RA) samples. Values are mean  $\pm$  SD. \*\*  $p < 0.01$ .

<https://doi.org/10.1371/journal.pone.0184469.g005>

( $p > 0.999$  for all). Hyperoxia treatment did not significantly alter total antioxidant capacity (88.8 vs 91.2%,  $p > 0.999$ ) (Fig 5).

## Discussion

BPD is a chronic lung disease experienced by prematurely born neonates, whose underlying pathophysiology includes inflammatory and oxidative stressors. In this study, we sought to investigate the molecular mechanisms by which these stressors communicate and potentiate damage, and have shown that the anti-inflammatory and anti-oxidant pathways interact in an *in vitro* macrophage model of BPD pathogenesis. We demonstrate how stimulation with LPS increases inflammation and reduces anti-oxidant gene expression, and how stimulation with hyperoxia increases anti-oxidant gene expression but only in the absence of LPS. These findings highlight the complex, multifactorial origins of BPD, contributing to our understanding of BPD pathogenesis.

Because inflammation and oxidative stress are important in BPD pathogenesis, we chose to study PPAR $\gamma$ , TNF $\alpha$ , Nrf2, Srxn1, and Hmox1. PPAR $\gamma$  has typically been studied in metabolic and cardiovascular diseases, but is also an important anti-inflammatory regulator due to its down-regulation of NF $\kappa$ B [14,15]. Additionally, addition of the PPAR $\gamma$  agonist rosiglitazone prevents hyperoxia-induced alveolar simplification [16–18], pulmonary inflammation [17], airway hyperreactivity [16], and pulmonary hypertension [18]. TNF $\alpha$  is a down-stream target of PPAR $\gamma$  and is the canonical pro-inflammatory cytokine implicated in a variety of diseases, including lung injury in preterm neonates [19].

Nrf2 is the master transcriptional regulator of the anti-oxidant response and is known to be important in lung disease, as mice deficient in Nrf2 experience higher mortality, have

increased pulmonary edema and inflammation [20,21], and develop pathologic changes similar to those found in BPD [8,22–29]. Furthermore, PPAR $\gamma$  and Nrf2 act in concert to protect the lung against hyperoxia-induced injury [8].

We have demonstrated that LPS produces a pro-inflammatory phenotype, as reflected by decreased expression of anti-inflammatory PPAR $\gamma$  and increased expression of pro-inflammatory TNF $\alpha$ . While this effect under room air (normoxia) is well reported in the literature [14,30,31], this study is the first *in vitro* study to our knowledge that has characterized the pro-inflammatory effect of LPS in BMDM under hyperoxic conditions. We have also shown that pro-inflammatory stimulation reduces the gene expression of anti-oxidant Nrf2 in both normoxic and hyperoxic environments and reduces total antioxidant capacity in normoxia, suggesting that inflammation reduces anti-oxidant defense.

Surprisingly, co-treatment with both LPS and hyperoxia did not result in a synergistic change in gene expression for most of our genes of interest. While hyperoxia increased gene expression of Srxn1, this was only observed in saline-treated BMDM; hyperoxia did not produce any significant increase in BMDM treated with LPS, and hyperoxia did not significantly alter total antioxidant capacity. We speculate, but have not confirmed, that this may be due to other antioxidant molecules which are not regulated by Nrf2, such as catalase [32]. Conversely, LPS treatment increased gene expression of Hmox1, but only in room air; LPS treatment under hyperoxia did not produce a significant change in gene expression. We speculate that the molecular basis for our observations may involve the Akt/PKB and NF $\kappa$ B pathways. It is known that LPS binding TLR4 activates Akt, which regulates antioxidant gene expression via Nrf2 [33], and that reactive oxygen species activate NF $\kappa$ B [34,35]. Our results may suggest a link between these important cellular signaling pathways.

One strength of our study is that we treated BMDM with LPS prior to hyperoxia exposure. Other studies of BPD have focused on one or the other, but in the clinical setting, antenatal infection, such as maternal chorioamnionitis, often precipitates preterm birth, and prematurely born neonates experience a relatively hyperoxic environment. This occurs due to the transition from relatively hypoxic intra-uterine life to the ambient extra-uterine environment, and is exacerbated by the administration of therapeutic supplemental oxygen [36]. Therefore, to model this clinical scenario, we treated BMDM with a pro-inflammatory stimulus two hours prior to placing them in a hyperoxic environment.

Another strength of our study is the investigation of cell culture conditions on studies of oxidative stress. We show that the BMDM media constituents have significant anti-oxidant activity, which may have protected the cells from hyperoxia and obscured significant changes in gene expression. We found that BMDM cultured in standard 10% FBS showed few changes in gene expression, but complete removal of FBS or L929-conditioned media resulted in cell death which precluded RT-PCR experiments. FBS has been shown to include the antioxidant enzyme superoxide dismutase [37] and may contain important antioxidant precursors, such as pyruvate, selenium [38], or alpha-tocopherol [39]. For example, a study in rodent astrogloma cells reported an effect of the serum content of preceding cell culture media on resistance to hydrogen peroxide, which persisted after changing to serum-free media [39]. Therefore, we believe the results from BMDM treated with reduced FBS more accurately reflect the cell's response to oxidative stress, rather than the response of the cell culture system.

Our study does have important limitations. While BMDM are important in the response to inflammation and oxidative stress, they are not identical to alveolar macrophages, the lung-resident macrophage. However, obtaining alveolar macrophages in sufficient quantities is difficult [11]. We also considered using peritoneal macrophages, but obtaining sufficient quantities would require recruitment such as via thioglycollate injection, which would induce a different phenotype than un-activated macrophages [40]. We chose BMDM because they

represent a well-established and well-studied model of the human immune system [10,41], and are easily obtained in abundant quantities. While other cell types are undoubtedly important in the pathogenesis of BPD, macrophages produce many signaling molecules implicated in the pathogenesis of BPD [6] and lie at the intersection of inflammation and oxidative stress [42–48]. An alternative approach of analyzing whole lung homogenates might be informative, but the heterogeneous cell population contained therein would make establishing the role of immune cells difficult to ascertain.

Second, our study is limited in its investigation of predominantly gene expression. Although protein level expression is important, for this study we sought to develop a rapid *in vitro* model in which to test possible therapeutics for BPD, and chose transcriptional regulation as the most accessible end point. We considered evaluating protein expression levels, but as PPAR $\gamma$  and Nrf2 are transcription factors, assaying nuclear translocation would be more technically challenging, while assaying total cell levels would be less informative. We did, however, evaluate levels of TNF $\alpha$  produced by BMDM in response to LPS and hyperoxia to validate our results.

Finally, we chose to study the effect of severe hyperoxia, 95% oxygen, which is not routinely used in current clinical practice. We did evaluate moderate hyperoxia as an oxidative stressor, and did not find any qualitative difference in cell survival or appearance between BMDM kept in room air, moderate hyperoxia, or severe hyperoxia. Therefore, to elicit the greatest response to an oxidative stressor, we used 95% hyperoxia in our model system.

In conclusion, we have established an *in vitro*, primary cell line-based model system with which we can investigate gene expression in response to inflammatory and oxidative stresses as relevant to BPD. We have demonstrated an interaction between the anti-inflammatory and anti-oxidant pathways with respect to gene expression, which we speculate may underlie altered host susceptibility to a “double hit” of infection and oxygen therapy. Future investigations into BPD pathogenesis should incorporate such dual-stimulation to more closely model the clinical scenario. Therapies that decrease the pro-inflammatory response or augment the anti-oxidant response may represent beneficial interventions for BPD.

## Supporting information

**S1 Table. Data.** Includes total antioxidant capacity values and  $\Delta Ct$  from Taqman RT-PCR (normalized to GAPDH) and TNF $\alpha$  values from Luminex.  
(XLSX)

## Acknowledgments

The authors are grateful to David Fletcher for technical support and performing Luminex assays, Lauren Auster and Kelly Goodrich for technical support, and to Dr. Richard Martin and Dr. Thomas Raffay for scholarly oversight and editorial assistance.

## Author Contributions

**Conceptualization:** Christopher R. Nitkin, Tracey L. Bonfield.

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**Funding acquisition:** Christopher R. Nitkin, Tracey L. Bonfield.

**Investigation:** Christopher R. Nitkin.

**Methodology:** Christopher R. Nitkin, Tracey L. Bonfield.

**Resources:** Tracey L. Bonfield.

**Supervision:** Tracey L. Bonfield.

**Writing – original draft:** Christopher R. Nitkin.

**Writing – review & editing:** Christopher R. Nitkin, Tracey L. Bonfield.

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